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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A01N 43/04, 63/00, 65/00, A61K 31/70, 48/00, C07H 21/04

A1

(11) International Publication Number: **WO 97/31532**

(43) International Publication Date: 4 September 1997 (04.09.97)

(21) International Application Number: PCT/US97/03244

(22) International Filing Date: 28 February 1997 (28.02.97)

(30) Priority Data:
08/608,132 28 February 1996 (28.02.96) US

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(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: ANTISENSE INTERLEUKIN 10 AND METHODS OF USE

(57) Abstract

The present invention pertains to a method for treating a patient with a disease in which the levels of interleukin-10 need to be down-regulated which comprises administering to the patient a therapeutically effective amount of an antisense oligodeoxynucleotide specific for interleukin-10 mRNA. The present invention also pertains to an antisense oligodeoxynucleotide specific for interleukin-10 mRNA having the formula 5'-TGGGTCTTGGTTCTCAGCTTGGGGCAT.

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ANTISENSE INTERLEUKIN 10 AND METHODS OF USE

BACKGROUND OF THE INVENTION

Statement of Rights to Inventions Made Under Federally-Sponsored Research and Development

Part of the work performed during development of this invention utilized United States Government funds. The United States Government has certain rights in this invention: NIH grant no. AI-29740 and a grant from the New Jersey Commission on Cancer Research.

Field of the Invention

The present invention pertains to a method for treating a patient with a disease in which the levels of interleukin-10 need to be down-regulated which comprises adminstering to the patient a therapeutically effective amount of an antisense oligodeoxynucleotide specific for interleukin-10 mRNA. The present invention also pertains to an antisense oligodeoxynucleotide specific for interleukin-10 mRNA having the formula 5'-TGGGTCTTGGTTCTCAGCTTGGGGCAT.

Description of the Background

The disclosures referred to herein to illustrate the background of the invention and to provide additional detail with respect to its practice are incorporated herein by reference and, for convenience, are numerically referenced in the following text and respectively grouped in the appended bibliography.

Growth Inhibition of Malignant CD5+B(B-1) Cells by Antisense Interleukin-10

The increased proliferation and longevity of malignant cells is, in part, due to increased production of, or response to, growth factors. Antisense oligodeoxynucleotides which interfere with mRNA translation of growth factors are an area of intensive investigation. Of particular interest is the role of antisense directed toward the cytokine interleukin-10 (IL-10), an important regulator of B cell growth and differentiation in malignant B-1 cells.

B-1 cells are thought to belong to a sub-population of B cells. These cells are usually of a larger size, CD5+ (B-1a) or CD5- (B-1b), according to the new nomenclature (1). Due to their long life span and self-renewal capabilities, B-1 cells have an increased ability to clonally expand and become malignant. The most striking example of malignant transformation of a B-1 cell is seen in human chronic lymphocytic leukemia (CLL), where the predominant malignant cell is a B-1 cell (2). Malignant clonal expansions of B-1 cells in aged NZB mice can serve as a model for human chronic lymphocytic leukemia (3, 4). NZB mice have similarities with chronic lymphocytic leukemia patients in that there is an age-dependent onset of clonal expansion of malignant B-1 cells, the indolent course, the presence of increased numbers of lymphocytes and "smudge" cells in the circulation, and the similar pathology with malignant cells infiltrating spleen, lymph nodes and bone marrow. In both the mouse model and patients with chronic lymphocytic leukemia, occasionally the malignancy can evolve into an aggressive diffuse large cell lymphoma termed Richter's syndrome (3). Both NZB malignant B-1 cells and human chronic lymphocytic leukemia cells have been found to express IL-10 mRNA (5-7). Genetic analysis of the NZB mice have revealed a strong correlation between high levels of IL-10 and B cell lymphoproliferative disease. IL-10 antisense was found to block cell growth in murine malignant NZB B-1 cells but have only a minimal effect on non-malignant B-1 cells (51).

IL-10 is a B cell growth regulatory cytokine that can be produced by many kinds of cells including Th2 cells, macrophages, monocytes, mast cells and B cells (8-11). Among murine B cells, B-1 cells are considered the main source of B cell derived murine interleukin-10 (mIL-10) (12) while in humans, B cells induced to produce human interleukin-10 (hIL-10) are associated with the mature B and preplasmocytic stages (13). IL-10 has been found to participate in the EBV transformation of human B cells and in the development of B cell lymphomas in

AIDS patients (56-58). IL-10 not only functions as a potent growth and differentiation factor for activated human B lymphocytes but it also plays a regulatory role on human monocytes (14, 15). IL-10 can function alone or together with other cytokines (15-17). In addition, exogenous IL-10 down regulates the production of IFN-*gamma* (15).

Gene sequencing has revealed that hIL-10 has homology to BCRF1 of Epstein-Barr virus (EBV) and the BCRF1 expressed protein has IL-10 activity (18). Furthermore, EBV transformed B lymphocytes constitutively secrete IL-10 and there is a relationship between IL-10 production by human malignant B cell lines and EBV expression (13, 19). Therefore, B cell transformation and abnormal proliferation is related to EBV infection and the consequent elevated IL-10 expression. Addition of viral IL-10 antisense prevented EBV induced B cell transformation (20). Evidence that IL-10 plays a role in malignant B cell transformation is found in AIDS associated B cell lymphomas in which increased hIL-10 has been found (19). Recently, the use of antisense in the treatment of diseases has been extensively investigated as a potential therapy (19-21). IL-10 antisense has been shown to inhibit the cell growth of non-Hodgkin's lymphoma from AIDS patients (21). Similarly, antisense IL-6 has also been shown to have growth inhibitory effects on cells from Hairy cell leukemia and various other malignancies, such as ovarian cancer, renal cancer, and myeloma cells (22-25).

Antisense Interleukin-10 Effects on Chronic Lymphocytic Leukemia Cell Growth

In the human malignancy, B-chronic lymphocytic leukemia (B-CLL), the malignant cell is a CD5+B cell. Morphological evidence as well as the phenotypic features of the circulating malignant cell in B-chronic lymphocytic leukemia suggest that the normal equivalent population is represented by resting CD5+B cells (43). In adult lymphoid tissues, CD5+B cells, or B-1 cells as they are referred to, are located at the edge of the germinal centers and within the mantle zone of secondary follicles. Like their normal B-1 counterparts, B-chronic lymphocytic leukemia cells coexpress CD5 and low levels of sIg, produce polyreactive antibodies and are capable of capping surface Ig. However, other features of B-chronic lymphocytic leukemia such as poor response to mitogenic stimuli, spontaneous proliferative responses to IL-2 and induction of apoptosis via Ig crosslinking are characteristic of an "activated" phenotype.

The increased proliferation and longevity of malignant cells is partly due to increased production of, or response to, growth factors. Additionally, failure to undergo programmed cell death may be due to abnormal expression of bcl-2 which plays a role in the accumulation of malignant CD5+B cells (44, 45). Consequently, antisense oligodeoxynucleotides that interfere with mRNA translation of growth factors, are candidates for therapy to regulate malignant cell growth.

SUMMARY OF THE INVENTION

The present invention pertains to a method for treating a patient with a disease in which the levels of interleukin-10 need to be down-regulated, such as diseases in which interleukin-10 is an autocrine growth factor for malignant cells or diseases wherein the inflammatory response is suppressed. The method for treating a patient with a disease wherein elevated levels of interleukin-10 has a detrimental effect, such as aquired inmmunodeficiency syndrome (AIDS), comprises adminstering to the patient a therapeutically effective amount of an antisense oligodeoxynucleotide specific for interleukin-10 mRNA.

The present invention also pertains to a method for treating a patient with chronic lymphocytic leukemia which comprises adminstering to the patient a therapeutically effective amount of an antisense oligodeoxynucleotide specific for interleukin-10 mRNA.

The present invention further pertains to an antisense oligodeoxynucleotide specific for interleukin-10 mRNA having the formula 5'-TGGGTCTTGGTTCTCAGCTTGGGGCAT.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a photograph illustrating a Southern blot of mInterleukin-10 and HPRT RT-PCR products from various cell lines.

Figure 2 is a graph illustrating inhibition of P12-L cell growth by Interleukin-10 antisense at various time points.

Figure 3 is a graph illustrating the effect of antisense Interleukin-10 exposure time on growth inhibition.

Figure 4 is a graph illustrating the dose dependent effects of Interleukin-10 antisense.

Figure 5 is a graph illustrating the effect of different oligos on malignant B-1 cell growth.

Figure 6 is a graph illustrating the effects of antisense on Interleukin-10 and IFN-*gamma* secretion by P12-L.

Figure 7 is a graph illustrating the responses of different cell lines to Interleukin-10 antisense. Panel A (upper) consisted of three different malignant B-1 cell lines which possessed high levels of Interleukin-10 mRNA and required Interleukin-10 for growth. Panel B (lower) consisted of five different cell lines, some of which expressed Interleukin-10 message and some of which did not.

Figure 8 is a graph illustrating the effects of rInterleukin-10 on malignant B-1 cell growth.

Figure 9 is a graph illustrating flow cytometric analysis of cells obtained from the peripheral blood of a patient with B-chronic lymphocytic leukemia. Panel B (upper) is a contour plot of unpurified PBL. Panel B (lower) is obtained following purification of B cells as described in the Materials and Methods.

Figure 10 is a graph illustrating representative data on growth inhibition following culturing of the PBL from an individual B-chronic lymphocytic leukemia patient.

Figure 11 is a graph illustrating the effect of exogenous addition of Interleukin-10 on the growth of malignant B-chronic lymphocytic leukemia cells.

Figure 12 is a graph illustrating the correlation between Interleukin-10 message levels (expressed as densitometric PCR values normalized to the expression of the housekeeping gene) and inhibition by antisense Interleukin-10

(expressed as control MTT values minus experimental) (panel A, upper) or the presence of CD5+B cells as determined by flow cyotmetric analysis (panel B, lower) in individual chronic lymphocytic leukemia samples.

DETAILED DESCRIPTION OF THE INVENTION

The present invention pertains to the use of antisense oligodeoxynucleotides specific for interleukin-10 mRNA in the treatment of patients with chronic lymphocytic leukemia. Interleukin-10 may be an autocrine growth factor for malignant B-1 cells and antisense therapy directed at interleukin-10 may be a potential tool in modulation of some B cell leukemias. Antisense interleukin-10 inhibits the growth of malignant B-1 cells and leads to cell death of malignant B-1 cells in some patients with chronic lymphocytic leukemia.

Malignant B-1 cells derived from NZB mice, a murine model of chronic lymphocytic leukemia, produce significantly higher levels of IL-10 mRNA than normal B-1 or B cells. IL-10 may act as an autocrine growth factor for malignant B-1 cells. By addition of antisense oligodeoxynucleotides specific for IL-10 mRNA, applicants were able to dramatically inhibit the growth of leukemic B-1 cells in a time and dose dependent manner. Control cell lines which do not depend on IL-10 for growth were not affected. Antisense therapy targeted at the 5' region of the IL-10 mRNA not only resulted in inhibition of malignant B-1 cell proliferation but also inhibited IL-10 production by malignant B-1 cells. Because endogenous IL-10 gene activation is critical for B-1 cell expansion, inactivation of the endogenous IL-10 gene by IL-10 antisense rather than extracellular regulation of the IL-10 gene product should be successful in controlling the malignant growth. From this NZB mouse model, applicants have established several *in vitro* cell lines that maintain the growth characteristics and cytokine profiles of the *in vivo* clones. Since both the *in vivo* and *in vitro* B-1 malignant cells had very high levels of IL-10 compared to non-malignant B or B-1 cells (3, 6), the use of IL-10 antisense to block cell growth was investigated.

The role of IL-10 on the *in vitro* growth of B cells from patients with B-chronic lymphocytic leukemia (CLL) was then investigated. In the present invention, peripheral blood cells from chronic lymphocytic leukemia patients were found to be varied in the amount of IL-10 mRNA present. Several chronic

lymphocytic leukemia samples underwent apoptosis in response to culturing in the presence of antisense IL-10. There was a correlation between the levels of IL-10 mRNA and the sensitivity to growth inhibition by antisense IL-10. This may indicate that antisense IL-10 inhibits cell growth in a sub-population of chronic lymphocytic leukemia in which IL-10 is an autocrine cytokine. An antisense therapy directed at the growth factors produced by malignant cells should be a way to control the growth of these cells. Therefore applicants proposed an antisense oligodeoxynucleotide (oligo) therapy to block the translation of IL-10 mRNA and inhibit the growth of malignant B-1 cells. As a result, applicants inhibited the growth of malignant B-1 cells *in vitro*. Therefore, IL-10 most likely plays a role as an autocrine growth factor for malignant B-1 cells and IL-10 antisense therapy could be a very promising tool in treating B cell leukemias.

Abbreviations used herein are: CLL, chronic lymphocytic leukemia; EBV, Epstein-Barr virus; oligo, oligodeoxynucleotide; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide); OD, Optical density; RT-PCR, reverse transcription-polymerase chain reaction; HPRT; hypoxanthine phosphoribosyl transferase; PBS, phosphate buffered saline; AS, antisense oligodeoxynucleotide; SS, sense oligodeoxynucleotide; CC, medium control; SO, scrambled oligodeoxynucleotide; rIL-10, recombinant murine IL-10; hIL-10, human IL-10; mIL-10, murine IL-10.

In a specific embodiment, the present invention is directed to a method for treating a patient with a disease in which the levels of interleukin-10 need to be down-regulated which comprises adminstering to the patient a therapeutically effective amount of an antisense oligodeoxynucleotide specific for interleukin-10 mRNA. Specifically, the disease may be a disease in which interleukin-10 is an autocrine growth factor for malignant cells or a disease in which the inflammatory response is suppressed. Preferably the disease is a disease in which interleukin-10 is an autocrine growth factor for malignant cells or is aquired inmmunodeficiency syndrome. Preferably, the malignant cells are B-1 cells and the disease is chronic lymphocytic leukemia. The antisense oligodeoxynucleotide specific for interleukin-10 mRNA may span the region adjacent to the initiation site of interleukin-10 translation, preferably region 1-500, more preferably region 310-347, and most preferably region 315-342. In a specific embodiment, the antisense oligodeoxynucleotide is 5'-TGGGTCTTGGTTCTCAGCTTGGGGCAT.

The present invention is further illustrated by the following examples which are not intended to limit the effective scope of the claims. All parts and percentages in the examples and throughout the specification and claims are by weight of the final composition unless otherwise specified.

Examples

Growth Inhibition of Malignant CD5+B (B-1) Cells by Antisense Interleukin-10

Cells Lines: NZB derived malignant B-1 cells:

P12-L and P13-L are aggressive malignant B-1 cell lines derived in our laboratory and serve as *in vitro* leukemic cell lines (11). P12-L was derived from cells of an (NZB X DBA/2) F1 mouse lymph node expanding a hyperdiploid B-1 clone which originated from an NZB. P12-L is CD5 negative and stromal cell dependent. P13-L, which is CD5+ and non-stromal cell dependent, was derived from a (NZB X DBA/2) F1 recipient of P12-L and possesses the same immunoglobulin as P12-L. Thus, P13-L is considered a sister cell line of P12-L (6). P12-L and P13-L have been carried *in vitro* for over one year. To avoid the possibility that the observed effects of IL-10 antisense were due to alterations which might have occurred during *in vitro* long-term culture, another freshly established malignant CD5+ B-1 cell line, 275T was also tested.

Control cell lines:

In order to assess whether the effect of antisense is specific, various control cell lines were employed in these experiments including B cells, fibroblasts, mast cells and T cells which have been previously shown to be capable of producing IL-10 or interacting with IL-10 (10, 11, 26). a) NYC is a B cell lymphoma cell line derived from an (NZB X NZW) Fl mouse and serves as a malignant B cell control. NYC was kindly supplied by Dr. Hans-Martin Jack, Loyola University, Chicago, IL. b) LNC-F is a stromal cell line that supports the growth of P12-L cells. P12-L cells are stromal cell dependent and readily undergo apoptosis when treated with anti-IgM. LNC-F is derived from the P12-L cell culture by repeatedly treating the P12-L cell culture with anti-IgM. LNC-F has been shown to be free of P12-L cells (6). c) P815 (from ATCC) is a mastocytoma cell line and is included as a control because IL-10 is a potential stimulatory factor for mast cells (11). d) T

cell controls included: EL-4 (from ATCC) is a thymoma cell line, and 3C2 is a cytotoxic T cell line generated in our laboratory that recognizes NZB malignant B-1 cells (including P12-L cells).

P12-L, P13-L, 275T, LNC-F, EL-4 and P815 were cultured in Iscove's modified Dulbecco's medium (IMDM) from GibcoBRL, Grand Island, NY; NYC and 3C2 were cultured in RPMI 1640 medium. All media supplemented with 10% heat inactivated FBS (Hyclone, Logan, Utah, USA.) and 100U/ml penicillin /streptomycin. In the case of 3C2, the media was supplemented by the addition of 10% Con A stimulated rat spleen cell supernatant (Con A-free) and 10 U/ml of mouse recombinant IL-2 (Genzyme, Cambridge, MA, USA.).

Oligodeoxynucleotides:

Based on the reported sequence of murine IL-10 (27), several antisense oligos were employed. IL-10 antisense I (ASI) and sense II (SSII) are the same sequences as primers used for IL-10 PCR (28). The IL-10 antisense oligos employed were chosen because they had a lack of homology to any other murine gene sequences reported in the GENBANK DNA database. The antisense/sense oligo pairs spanned regions close to the initiation site of IL-10 translation rather than the termination site (which occurs at residue 610). IL-10 antisense I (ASI) (5'-CATTTCCGATAAGGCTTGG) and sense I (SSI) (5'-CCAAGCCTTATCGGAAATG) (regions 315-333); IL-10 antisense II (ASII) (5'-CGATTATTGTCTTCCCG) and sense II (SSII) (5'-CGGGAAGACAATAACTG) (regions 147-163); and a scrambled oligo (SO), (5'-TTTATTGCGCACGACGGAT) which has the same base content of IL-10 ASI were used. All oligos were synthesized by Operon Technologies Inc. (Alameda, CA, USA). Oligos were diluted in medium and added at concentrations indicated at the beginning (time 0) of culture. For the study of the effects of exogenous IL-10, recombinant murine IL-10 (rIL-10), (PharMingen, San Diego, CA, USA.) was added at time 0 with or without oligos at concentrations indicated.

MTT assay:

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) quantitative colorimetric assay was used to detect living, but not dead cells and the signal generated is dependent on the degree of activation of the cells and thus can be used to measure cytotoxicity, proliferation or activation (29). In

order to detect the cellular growth and survival, MTT assay was performed according to published techniques with slight modifications (30). Briefly, cells (1 X 10^4/well) were cultured with or without oligos for various times, then 20 μl of MTT (5mg/ml in PBS, Sigma, St. Louis, MO, USA) were added and the cells cultured in 37°C for an additional 4 hours. In some experiments, cultures were exposed to oligos for a specified time followed by centrifugation, washing 2X and resuspension in fresh media. In these experiments, sense, antisense and media control groups were handled in a similar manner. The culture supernatants were gently flicked out and 150 μl of PBS were added and the plates were centrifuged for 5 minutes at 2000 rpm. The PBS was removed. 100 μl of 100% 2-propanol were added and vigorously pipetted to dissolve the formazan crystals. Plates were stored in dark for 30 minutes at room temperature and read at 570nm with a reference at 650nm on a Kinetic Microplate reader (Molecular Devices, Menlo Park, CA, USA). Optical density (OD) was determined and background values subtracted.

Quantification of cytokine message levels by PCR analysis:

Total RNA was prepared using RNAzol B according to the manufacturer's specifications (TEL-Test, Friendswood, TX, USA). Reverse transcription and PCR were performed according to the manufacturer's instructions included with the Perkin Elmer Cetus GeneAmp RNA PCR kit (Perkin Elmer Cetus Corp., Norwalk, CT, USA). All primers and probes were synthesized by Operon Technologies Inc. (Alameda, CA, USA). IL-10 and HPRT primers and probes employed have previously been described (28). PCR sample quality was examined on a 2% NuSieve agarose gel (FMC Bioproducts, Rockland, ME, USA) using ethidium bromide and transferred to nitrocellulose (Schleicher and Schuell, Keene, NH, USA) and probed with ^{32}P-labeled internal oligo probes, which hybridized to a portion of the amplified segment between the nucleotide sequences complementary to the primers.

ELISA for Interleukin-10 and IFN-*gamma* Detection

P12-L cells (1 X 10^4 cell/ml) were incubated with either antisense or sense oligos, or medium alone for 24, 48, and 72 hours. After each time interval, supernatants were collected, concentrated (5X) and measured for IL-10 and IFN-*gamma* levels using a murine IL-10 ELISA kit from Endogen (Boston, MA, USA) and a murine IFN-*gamma* ELISA kit from Genzyme (Cambridge, MA, USA). The IL-10 ELISA utilized was capable of detecting a minimum of 0.14 U/ml of IL-10.

The IFN-*gamma* ELISA utilized was capable of detecting a minimum of 125 pg/ml of IFN-*gamma*. Known positive and negative controls were employed. Due to the limited level of detection of the ELISA kits and the low number of the cells in culture (1 X 10^4/ml), 5 times concentrated supernatants of the culture and medium alone for control were employed.

Figure 1 is a photograph illustrating a Southern blot of mIL-10 and HPRT RT-PCR products from various cell lines. RNAs from various cell lines studied were extracted using RNAzolB according to the manufactures protocol. An RT-PCR amplification was carried out with primers specific for mIL-10 and HPRT using an RT-PCR kit. PCR products were examined by Southern blot analysis using mIL-10 and HPRT specific ^{32}P labeled internal oligo probes. Equal amounts of mIL-10 and HPRT PCR products were used and the detection of HPRT is for semiquantitative purposes.

Figure 2 is a graph illustrating inhibition of P12-L cell growth by IL-10 antisense at various time points. P12-L cells were cultured (1 X 10^4 cell/well) in IMDM medium with 20μM IL-10 antisense (ASI) or sense (SSI). Cells cultured in medium alone (CC) were also used as a control. Cells were cultured at 37°C for different time intervals as indicated. MTT was added for another 4 hours. Afterwards, cultures were washed with PBS and blue formazon crystals were dissolved with 100% 2-propanol. ODs were read at 570nm with 650nm reference and background subtracted. The results are expressed as mean ± SD of two individual experiments.

Figure 3 is a graph illustrating the effect of antisense IL-10 exposure time on growth inhibition. P12-L cells (1 X 10^4 cells/well) were exposed to either 20μM sense, antisense or media alone for 24, 48, or 72 hours. Following the specified exposure time the cells were washed and resuspended in fresh media. The MTT assay was performed at 96 hours for all groups. The results are expressed as % inhibition of MTT values of sense and antisense treated cultures relative to the appropriate media control MTT values. The results are expressed as mean ± SD of two individual experiments.

Figure 4 is a graph illustrating the dose dependent effects of IL-10 antisense. P12-L cells were cultured (1X10^4 cell/well) in IMDM medium with different concentrations of IL-10 antisense (ASI) and sense (SSI) as indicated. Cells cultured in medium alone (CC) were also used as a control. After 72 hours of

incubation at 37°C, MTT assay was performed as described above. The results are expressed as mean ± SD of two individual experiments.

Figure 5 is a graph illustrating the effect of different oligos on malignant B-1 cell growth. P12-L cells (1 X 10^4 cells/well) were cultured with two antisense oligos (ASI, ASII) targeting at different regions of the IL-10 mRNA. As a control, medium alone and two corresponding sense oligos (SSI, SSII) and a scrambled oligo (SO) consisting of the same A,T,G,C content of ASI were employed. All oligos were used at 20μM. After 72 hours, cell growth was measured by a MTT colorimetric assay. The results are expressed as mean ± SD of two individual experiments.

Figure 6 is a graph illustrating the effects of antisense on IL-10 and IFN-*gamma* secretion by P12-L. P12-L cells (1 X 10^4 cell/ml) were incubated with 20μM ASI, SSI or medium alone (CC) for 24, 48 and 72 hours. After each time interval, supernatants were collected, concentrated (5X) and measured for mIL-10 and IFN-*gamma* levels by murine IL-10 and IFN-*gamma* ELISA kits, as described above. The results are expressed as mean ± SD of three individual experiments.

Figure 7 is a graph illustrating the responses of different cell lines to IL-10 antisense. Different cell lines were cultured at 1 X 10^4 cell/well with 20μM ASI, SSI or medium alone. After 72 hours of incubation at 37°C, MTT assay was performed as described above. Panel A (upper) consisted of three different malignant B-1 cell lines which possessed high levels of IL-10 mRNA and required IL-10 for growth. Panel B (lower) consisted of five different cell lines, some of which expressed IL-10 message and some of which did not. However, all of the cell lines in panel B did not require IL-10 for growth. The results are expressed as mean ± SD of two individual experiments.

Figure 8 is a graph illustrating the effects of rIL-10 on malignant B-1 cell growth. P12-L cells (1 X 10^4 cells/well) were cultured with 20μM ASI, SSI or medium alone and varying concentrations of rIL-10 (0-50 ng/ml) for 72 hours. Afterwards, cell growth was measured using a MTT assay as described above. The results are expressed as mean ± SD of two individual experiments.

Antisense Interleukin-10 Effects n Chr nic Lymph cytic Leukemia Cell Growth

Chronic Lymphocytic Leukemia Cells:

Lymphocytes from heparinized blood of human B-chronic lymphocytic leukemia patients or age-matched controls were obtained following Ficoll-Hypaque separation. In some cases B cells were further purified by depletion of monocytes by using L-leucine methyl ester (l-LME) and of T cells by rosetting with neuraminidase treated sheep red blood cells. Both before and after purification, samples that were tested, were stained with selected monoclonal antibodies, CD5 and CD19 (Caltag, San Francisco, CA, USA) and analyzed by flow cytometric techniques on a FACscan (Becton Dickinson, Sunnyvale, CA, USA). Cells (5 X 10^4/well) were cultured in RPMI 1640 (GibcoBRL, Grand Island, NY, U.S.A.). All media was supplemented with 10% heat inactivated FBS (Hyclone, Logan, Utah, USA.) and 100U/ml penicillin /streptomycin.

Oligodeoxynucleotides:

The sequence of human IL-10 which used as the antisense oligo, was based on the a similar sequence successfully employed in the murine situation (51). The IL-10 antisense oligos employed lack of homology to any other human gene sequences reported in the GENBANK DNA database. The antisense/sense oligo pairs spanned regions close to the initiation site of IL-10 translation rather than the termination site (which occurs at residue 610). IL-10 antisense (AS) (5'-TGGGTCTTGGTTCTCAGCTTGGGGCAT) and sense (SS) (5'-ATGCCCCAAGCTGAGAACCAAGACCCA) (regions 315-342) were thus chosen. All oligos were synthesized by Operon Technologies Inc. (Alameda, CA, USA). Oligos were diluted in medium and added at 20μM concentrations at the beginning (time 0) of culture.

Exogenous Interleukin-10:

For the study of the effects of exogenous IL-10, recombinant hIL-10 (rIL-10) at 50 ng/ml and 100 ng/ml (Schering Plough, Kenilworth NJ, USA.) was added at time 0.

MTT assay:

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) quantitative colorimetric assay was used to detect living, but not dead cells and the signal generated is dependent on the degree of activation of the cells. This can be used to measure cytotoxicity, proliferation or activation. In order to detect the cellular growth and survival, the MTT assay was performed according to published techniques with slight modifications (66). Briefly, cells (5 X 10^4/well) were cultured with or without oligos for various times, then 20 μl of MTT (5mg/ml in PBS, Sigma, St. Louis, MO, USA) were added and the cells cultured at 37°C for an additional 4 hours. The supernatants were gently flicked out and 150μl of PBS were added and the plates were centrifuged for 5 minutes at 2000 rpm. After removal of PBS, 100μl of 100% 2-propanol were added and vigorously pipetted to dissolve the formazan crystals. Plates were stored in dark for 30 minutes at room temperature and read at 570nm with a reference at 650nm on a Kinetic Microplate reader (Molecular Devices, Menlo Park, CA, USA). Optical density (OD) was determined and background values subtracted.

Quantification of cytokine message levels by PCR analysis:

Total RNA was prepared using RNAzol B according to the manufacturer's specifications (TEL-Test, Friendswood, TX, USA). Reverse transcription and PCR were performed according to the manufacturer's instructions included with the Perkin Elmer Cetus GeneAmp RNA PCR kit (Perkin Elmer Cetus Corp., Norwalk, CT, USA). All primers and probes were synthesized by Operon Technologies Inc. (Alameda, CA, USA). IL-10 and b-actin primers employed have previously been described (53, 67). PCR sample quality was examined on a 2% NuSieve agarose gel (FMC Bioproducts, Rockland, ME, USA) using ethidium bromide and analyzed using ImageQuant software (Molecular Dynamics, San Francisco, CA, U.S.A.). The densitometric values for the IL-10 PCR products are expressed as the ratio of IL-10 PCR product/b-actin PCR product in order to normalize for the amount of RNA present in each sample.

Anti-IgM treatment of cultures:

In order to induce apoptosis and reduce viability by a method independent of antisense IL-10, separate cultures were exposed from time 0 with anti-IgM at 8μg/ml (Sigma).

Figure 9 is a graph illustrating flow cytometric analysis of cells obtained from the peripheral blood of a patient with B-chronic lymphocytic leukemia. Histograms represent profiles obtained following dual color staining of cells with anti-CD19 FITC and anti-CD5 PE. Panel B (upper) is a contour plot of unpurified PBL. Panel B (lower) is obtained following purification of B cells as described above.

Figure 10 is a graph illustrating representative data on growth inhibition following culturing of the PBL from an individual B-chronic lymphocytic leukemia patient. MTT assay was performed at 24, 48 and 72 hours. The results are expressed as % inhibition of MTT values of anti-IgM, sense IL-10 and antisense IL-10 treated cultures relative to the appropriate media control MTT values. The results are expressed as mean ± SD.

Figure 11 is a graph illustrating the effect of exogenous addition of IL-10 on the growth of malignant B-chronic lymphocytic leukemia cells. All chronic lymphocytic leukemia cells were cultured in the presence of 100 ng/ml of IL-10. After 72 hours of incubation at 37°C, MTT assay was performed as described above. The results are expressed as mean ± SEM.

Figure 12 is a graph illustrating the correlation between IL-10 message levels (expressed as densitometric PCR values normalized to the expression of the housekeeping gene) and inhibition by antisense IL-10 (expressed as control MTT values minus experimental) (panel A, upper) or the presence of CD5+B cells as determined by flow cyotmetric analysis (panel B, lower) in individual chronic lymphocytic leukemia samples.

Results

Growth Inhibition of Malignant CD5+B (B-1) Cells by Antisense Interleukin-10

Quantification of mIL-10 mRNA by RT-PCR of Various Cell Lines

An RT-PCR method was employed to detect the mIL-10 mRNA present in various cell lines studied. This technique is semiquantitative and primers specific for the housekeeping gene, HPRT, are employed to verify that

approximately the same amount of RNA is subjected to RT/PCR in all the samples analyzed. As shown in Figure 1, 3C2, P815, and EL-4 showed no detectable IL-10 mRNA while NYC and LNC-F showed a low and moderate IL-10 mRNA level respectively. IL-10 is not a growth factor for all the cell lines mentioned above. In contrast, malignant B-1 cells (P12-L and P13-L) not only required IL-10 as a growth factor, but also possessed high levels of IL-10 mRNA. This unique characteristic formed the basis of IL-10 antisense therapy for malignant B-1 cells.

Growth Inhibition Effect of Interleukin-10 Antisense

The NZB derived malignant B-1 cell line, P12-L was found to possess high levels of IL-10 mRNA (6). For the study of growth regulation, this cell line was studied for the effect of IL-10 antisense on the growth inhibition. The results (Figure 2) demonstrated that the growth of the malignant B-1 cell line, P12-L, is significantly inhibited by IL-10 antisense oligodeoxynucleotides (IL-10 ASI). The inhibition rates are 49.6%, 80.1%, 94.9% at 24, 48, and 72 hours, respectively. The maximal inhibitory effect of IL-10 antisense was achieved at 72 hours. Contrary to the antisense treatment group, the sense and control treatment groups showed a steady rise in cell number as the time intervals increased from 24 to 72 hours. Similar results were obtained for P12-L cells cultured in the presence of 20 μM IL-10 antisense for longer periods of time, 4 days and 7 days. Greater than 95% reduction in MTT was observed at these later time points when compared to media control. In contrast, no difference was observed between media control and sense treated cultures at these later time points. Trypan blue exclusion studies indicated that in cultures treated with antisense IL-10, by Day 4 greater than 95% of the cells took up the dye. Likewise on Day 7, more than 95% of the cells were dead following IL-10 antisense treatment.

Analysis by light microscopy indicated that the few remaining viable cells following IL-10 antisense treatment were exclusively slow-dividing stromal cells in contrast to the sense treated wells in which the rapidly dividing P12-L cells were the majority of viable cells. Electron micrographs of P12-L cells (data not shown) at 48 hours post-antisense treatment confirmed that most of the remaining viable cells had stromal cell morphology and the dead lymphoid cells had apoptotic features.

Additional experiments were performed to determine the minimal amount of time cells must be exposed to antisense in order to result in inhibition of

growth. Washout experiments were performed in which P12-L cells were exposed to either antisense, sense or media alone for a specified time (Figure 3). The cells were then washed and re-cultured in fresh media for a total of 96 hours of culture. Even following only 24 hours of initial exposure to antisense IL-10, significant growth inhibition was observed at the 96 hour assay time point. There was little difference observed between cells cultured in the presence of IL-10 antisense for only 48 hours and those cells exposed to antisense for the entire culture period.

Dose Dependence of Interleukin-10 Antisense

Due to the limited cell uptake and the rapid degradation of oligos, a sufficient dose of oligos would be required. As shown in Figure 4, IL-10 antisense inhibited the growth of P12-L cells in a dose dependent manner. As the IL-10 antisense oligo concentrations increased from 5 to 40 μM, the inhibitory effect increase rapidly, and maximal inhibitory effect of antisense oligo was observed at 40μM. Concentrations less than 5μM of antisense showed no notable effects. On the other hand, the corresponding sense oligo showed no significant effects on P12-L cell growth at all concentrations.

Effects of different oligodeoxynucleotides derived from the Interleukin-10 sense or antisense sequence

In order to determine the specificity of the inhibitory effect of IL-10 antisense, several different oligos were employed. As shown in Figure 5, another IL-10 antisense (ASII, closer to the 5' region than ASI) showed similar inhibitory effects. The corresponding sense oligo (SSII) had no effects on malignant B-1 cell growth as had previously been observed for SSI. A scrambled oligo consisting of the same A,T,G,C content of ASI was also employed. In contrast to the antisense oligo, the scrambled oligo showed no inhibitory effect on the growth of malignant B-1 cells.

Effects of Interleukin-10 Antisense on the Production of Interleukin-10 and IFN-*gamma*

The P12-L cell line has been extensively analyzed (6). Since P12-L cells express high levels of IL-10 mRNA and are exquisitely sensitive to IL-10 antisense treatment, the cytokine protein levels were studied in this line. P12-L

cells can spontaneously secrete IL-10. Upon the IL-10 antisense entry into the cells, IL-10 translation is expected to be interrupted. The secretion of IL-10 in antisense treated and untreated groups was measured by ELISA. As shown in Figure 6, the IL-10 in the supernatants of the antisense treated group remained at the background level, while the IL-10 in both the sense treated group and the control group increased markedly with time in culture. The IL-10 levels of both sense and control groups were similar. Since human chronic lymphocytic leukemia cells have been reported to produce IFN-*gamma* (31) and IL-10 can inhibit the production of IFN-g (32), the effects of IL-10 antisense treatment on the malignant cells in terms of the production of IFN-*gamma* was investigated. The levels of IFN-*gamma* in the supernatants of IL-10 antisense treated cell cultures were assayed. No increase of IFN-*gamma* in culture supernatants of antisense treated or sense treated group was detected. None of the concentrated culture supernatants (antisense treated, sense treated and control) showed detectable levels of IFN-*gamma* (Figure 6).

Effects of Interleukin-10 Antisense on Various Cell Lines

The inhibitory effects of IL-10 antisense oligos were not observed in all cell lines studied. Individual cell lines displayed different responses to IL-10 antisense (Figure 7). The growth of those malignant B-1 cell lines (P12-L, P13-L, 275T) which possessed elevated levels of IL-10 mRNA were significantly inhibited in the presence of IL-10 antisense. There is a significant growth inhibition effect when compared to the growth of sense oligo treated and medium control groups. In contrast, the growth of the control cell lines (EL-4, NYC, P815, 3C2 and LNC-F) was not affected by IL-10 antisense. No significant differences among the control cell lines was noted between the antisense, sense, and medium groups. For all the control cell lines, IL-10 is not a required growth factor, although some of the control lines possessed IL-10 mRNA (Figure 1).

Failure to Reverse Inhibitory Effect of Interleukin-10 Antisense by the Addition of Exogenous Interleukin-10

If IL-10 is an autocrine growth factor for malignant B-1 cells, it is reasonable to postulate that addition of exogenous IL-10 should enhance the growth of these cells and reverse the inhibitory effect of IL-10 antisense. However, in our experiments the addition of rIL-10 neither enhanced the growth of malignant B-1 cells, nor reversed the growth inhibitory effect of IL-10 antisense, even at high

concentrations up to 50ng/ml (25 fold higher than physiological IL-10 levels) (Figure 8).

Antisense Interleukin-10 Effects on Chronic Lymphocytic Leukemia Cell Growth

Surface expression of CD5 on Chronic Lymphocytic Leukemia cells:

Dual staining analysis of PBL from an individual B-chronic lymphocytic leukemia patient is shown in Figure 9. This patient had a population of homogeneously staining cells which were CD19+ and expressed low levels of CD5 (Figure 9A). On further purification the cells were found to be exclusively CD5+B cells with <1% residual T cells (Figure 9B). The proportion of CD5+B cells in the PBL varied in the six patients studied from 20-87%.

Growth Inhibitory Effect of Interleukin-10 Antisense:

Six chronic lymphocytic leukemia samples were cultured for three days in complete medium with either no additives or 20μM sense IL-10, 20μM antisense oligonucleotides or 8μg anti-IgM. At intervals of 24, 48 and 72 hours, the cells were assayed for viability by the MTT assay (Table 1). Anti-IgM was employed as a known agent which induces apoptosis in malignant B-1 cells (68, 69). Sense IL-10 oligodeoxynucleotides did not have any significant effect on the viability of the cells when compared to cultures in which no additives were employed (control). In contrast, at 72 hours, the antisense IL-10 showed significant inhibition of viability in 3/6 patients studied. The inhibitory effect of antisense IL-10 was observed to occur in a time dependent manner as seen in a representative sample (Figure 10). The maximal inhibitory effects of IL-10 antisense and anti-IgM were achieved at 72 hours. Contrary to the antisense treatment group, the sense treatment group showed a steady rise in cell number as the time intervals increased from 24 to 72 hours. Control PBL from age-matched non-chronic lymphocytic leukemia normals showed no growth inhibition by any of the additives employed. Unfortunately, it was impossible to obtain enough purified CD5+B cells from normal PBL to absolutely determine that antisense-IL-10 had no inhibitory effect on B-1 cells. However, based on the results using unpurified PBL in non-chronic lymphocytic leukemia controls, antisense IL-10 and anti-IgM had no inhibitory effects. The overall effects of antisense IL-10, sense IL-10 and anti-IgM are summarized in Table 1.

Table 1

Effect of Antisense Interleukin-10 on B-Chronic Lymphocytic Leukemia Cells

Effects of	Sense	Antisense IL-10	Anti IgM
	88*	36	41
	109	84	23
	115	83	64
	103	116	56
	93	101	72
Average	101.6	84	51.2
St. Dev.	11.6	30.1	19.4

* Results are expressed as percentages of control values.

Effect of Exogenous Interleukin-10 :

Previous investigators have reported that IL-10 itself can be inhibitory for malignant B-1 cells (70). However, in our experiments the addition of rIL-10 neither enhanced nor inhibited the growth of malignant B-1 cells (Figure 11).

Quantification of Interleukin-10 mRNA by RT-PCR:

An RT-PCR method was employed to detect the IL-10 mRNA present in various B-chronic lymphocytic leukemia samples. This technique is semiquantitative and primers specific for the housekeeping gene, b-actin, are employed to verify that approximately the same amount of RNA is subjected to RT/PCR in all the samples analyzed. The growth inhibitory effect of antisense IL-10 was directly related to the presence of detectable levels of IL-10 mRNA (Figure 12A). In the absence of IL-10 mRNA expression, antisense IL-10 was ineffective.

However, there was no significant correlation between the levels of CD5+B cells in the patients and the levels of IL-10 mRNA (Figure 12B).

Discussion

Growth Inhibition of Malignant CD5+B (B-1) Cells by Antisense Interleukin-10

Antisense therapy against cytokines may be a potential therapeutic tool for the treatment of a variety of disease states. For instance, IL-6 antisense has been employed for the treatment of renal cell carcinoma, myeloma and ovarian cancer; IL-1 antisense for the treatment of inflammatory disease and IL-11 antisense in the treatment of malignant megakaryoblastic cells (22-25, 33, 34). In this report, the effects of IL-10 antisense on malignant B-1 cells were studied.

Although IL-10 has only been identified within the last 3 years, it has been the subject of numerous investigations (8, 10, 12, 14-17, 35). Studies have suggested that IL-10 is an autocrine growth factor for B-1 cells (8, 14) and functions as a potent growth and differentiation factor for activated human B lymphocytes. In humans, B lymphomas possessed elevated levels of IL-10 which was also related to the infection by EBV (13, 19). Masood, et al (21) reported that IL-10 is an autocrine B-cell growth factor for human B cell lymphoma and their growth can be blocked by IL-10 antisense. Similarly, applicants have reported that murine malignant B-1 cells possess high levels of IL-10 mRNA (5, 6, 36). Therefore applicants speculated that the elevated IL-10 in murine malignant B-1 cells may contribute in part to their rapid growth. An IL-10 antisense treatment was designed and *in vitro* culture studies performed. The results of these experiments indicated that the IL-10 antisense oligo not only inhibited the growth but also lead to cell death of malignant B-1 cell lines studied. These results showed that IL-10 is required for both the growth and survival of malignant B-1 cells studied. However, similar to the study of IL-6 antisense reported by Watson (25), the addition of exogenous rIL-10 neither stimulated the growth of these malignant B-1 cells nor reversed the inhibitory effect on these cells of IL-10 antisense oligo. One possibility is that the effect of IL-10 antisense is nonspecific. This seems unlikely based on the results of several additional experiments. Two IL-10 antisense oligos targeted at different region of the IL-10 mRNA showed similar inhibitory effects, while the sense and the scrambled oligos showed no notable effects. Furthermore, the IL-10 antisense did not have any nonspecific inhibitory

effects on several other cell lines. Even in the stromal cell line (LNC-F), which expressed moderate amounts of IL-10, cell growth was not inhibited by IL-10 antisense. The role of IL-10 for the stromal cell is unclear, but it does not seem to be a growth factor required for these cells. There does not appear to be a correlation between the level of IL-10 mRNA expressed and the susceptibility to growth inhibition by IL-10 antisense. A more important criterion for susceptibility to inhibition by IL-10 antisense may be the potency of IL-10 as a requisite endogenous growth factor. The fact that the addition of exogenous IL-10 did not enhance the growth of malignant B-1 cells also indicated that endogenous high levels of IL-10 seem to be required for the growth of malignant B-1 cells. Our results suggested that only endogenous IL-10 production can achieve the levels that are required for the growth of these malignant cells and the autocrine requirement could not be substituted by IL-10 replacement therapy. Since endogenous IL-10 gene activation is critical for B-1 cell expansion, inactivation of the endogenous IL-10 gene by IL-10 antisense rather than extracellular regulation of the IL-10 gene product should be more successful in controlling malignant growth.

The effect of IL-10 antisense was manifested in a time and dose dependent manner. As reported by others (37, 38), the effect of antisense increased with time. In our experiment, the inhibitory effects were maximal 72 hours after addition of the IL-10 antisense oligos to culture. Sufficient time may be required for the cells to take up the antisense oligos and for the translation of IL-10 protein to be reduced. In addition, enough time must be allowed for the cell to use up any IL-10 formed prior to the addition of antisense oligos. The results of experiments in which IL-10 antisense was removed at various time points during culture suggest the antisense need only be present in the cultures for the initial 48 hours to reach near peak inhibition observed at 72 hours. Not only is there a time dependence for antisense IL-10 growth inhibition, there is also a dose dependence of antisense. As shown in Figure 4, sufficient amount of IL-10 antisense is also required for growth inhibition effect ($>5\mu M$). This may be due to the rapid degradation and the low efficiency of cellular uptake of the unmodified oligos employed. Phosphorothioate modified antisense oligos which are more resistant to degradation and *in vivo* experiments are being conducted in our laboratory. Applicants have reported that malignant B-1 cells of NZB mice undergo apoptosis induced by anti-IgM antibodies (39). Applicants observed that the P12-L malignant B-1 cells also undergo apoptosis after being treated with IL-10 antisense oligos for 48 hours. This may indicate that both a strong stimulation via cross linking the antigen receptor or a

lack of growth factor will lead the malignant cells to die by a programmed form (apoptosis).

Evidence suggests that a cytokine network regulates the production of various cytokines. The production of IFN-*gamma* is inhibited by IL-10 (32). It has been reported that human chronic lymphocytic leukemia cells which are very slow growing express detectable IFN-*gamma* mRNA and IFN-*gamma* can induce proliferation and differentiation in B-chronic lymphocytic leukemia cells (31, 40). However, this is not the case in our experiment system. The aggressive murine malignant B-1 cells studied herein do not possess detectable IFN-*gamma* mRNA as assayed by PCR (6). Our recent experiments have also shown that the IL-10 antisense treatment does not lead to the production of IFN-*gamma* at any time point studied (up to 72 hours) as detected by ELISA. In addition, culture supernatants from the IL-10 antisense treated cells do not accelerate or block the growth of the untreated cells (data not shown). IFN-*gamma* has also been shown to be an inhibitory factor for the growth of CD5+B cells (41). There is a possibility that if IL-10 prevents the production of IFN-*gamma* by the malignant B-1 cells, the decreased growth of the malignant B-1 cells following the IL-10 antisense treatment is due to the increased IFN-*gamma* production which may then exert the inhibitory effect. However, no detectable IFN-*gamma* mRNA and protein may exclude the possibility that the growth inhibition observed by the IL-10 antisense treatment is due to the IFN-*gamma* production.

Several viruses have genes with homology to IL-10 (27, 42). The EBV has a gene BCRFI with homology to the human IL-10 gene and infection with this virus results in the immortalization of B cells and the overexpression of B-cell derived endogenous IL-10 (13, 19). The increased production of IL-10 may be responsible for the growth and/or development of some human B cell malignancies. Antisense IL-10 treatment may have a role in reducing viral effects and abnormal B cell proliferation.

In summary our results showed that IL-10 may be an autocrine growth factor for malignant B-1 cells and that antisense therapy directed at IL-10 may be a potential tool in modulation of B cell leukemias.

Antisense Interleukin-10 Effects on Chronic Lymphocytic Leukemia Cell Growth

In this report, applicants found that a subpopulation of all B-chronic lymphocytic leukemia patients respond to antisense-IL-10 by demonstrating a significant decrease of cell growth in culture. These responding chronic lymphocytic leukemia patients are similar to the NZB mouse model in which all the B-1 malignant cells produce high levels of IL-10. In this murine model, applicants have found that in addition to an *in vitro* inhibitory effect of antisense IL-10, *in vivo* prolonged antisense IL-10 administration also prevented malignant B-1 cell growth (51). Antisense therapy against cytokines may be a potential therapeutic tool for the treatment of a variety of disease states. For instance, IL-6 antisense has been employed for the treatment of renal cell carcinoma, myeloma and ovarian cancer; IL-1 antisense for the treatment of inflammatory disease and IL-11 antisense in the treatment of malignant megakaryoblastic cells (62-65, 71, 72) In addition to cytokine antisense therapy, bcl-2 has also been targeted for antisense therapy (73)

In this report and previous reports (53, 74), the levels of IL-10 mRNA in malignant B-1 cells were found to be varied. Previous investigators have reported a variety of cytokines to be elevated in B-chronic lymphocytic leukemia (75-78). Cytokines not only promote the growth of B-1 malignant cells but can also regulate their growth (43, 75). Other investigators (70) have found that exogenous IL-10 was inhibitory for B-chronic lymphocytic leukemia cell, however, under the experimental conditions employed in this report applicants were unable to find any apoptotic effects of IL-10 over a wide concentration range (10-100ng/ml) tested. In the current study, IL-10 mRNA was found to be elevated in only some chronic lymphocytic leukemia patients. It is not surprising that there was a correlation between growth inhibition by antisense IL-10 in the B-chronic lymphocytic leukemia patients and the level of IL-10 message. It appears that only in the situation in which IL-10 is an autocrine growth factor can growth inhibition by IL-10 antisense be effective.

In our analysis of the murine model of chronic lymphocytic leukemia, applicants have found that IL-10 plays a central role in the development of B-chronic lymphocytic leukemia like malignancies. Previous studies in mice have shown that IL-10 is not required for the development of B-1 cells (79). However in NZB mice, B-1 malignant growth does seem to be dependent upon IL-10 levels. In the current study, applicants found that increased CD5+B cells in the

peripheral blood, which is a hallmark of chronic lymphocytic leukemia, was partially correlated to the levels of IL-10. Perhaps, other cytokines in addition to IL-10 can provide growth signals for B-1 cells. Cytokines may not solely be responsible for malignant B-1 development and growth, other genetic alteration involving oncogenes, such as bcl-2, may be required for malignant B-1 cell development.

Although IL-10 has only been identified within the last 3 years, it has been the subject of numerous investigations (54). Studies have suggested that IL-10 is an autocrine growth factor for B-1 cells (54, 59) and functions as a potent growth and differentiation factor for activated human B lymphocytes. In humans, B lymphomas possessed elevated levels of IL-10 which was also related to infection by EBV (56, 80). Masood, et al. (61) reported that IL-10 is an autocrine B-cell growth factor for human B cell lymphoma and their growth can be blocked by IL-10 antisense. Similarly, applicants have reported that murine malignant B-1 cells possess high levels of IL-10 mRNA and are susceptible to antisense IL-10 inhibition (51, 52, 81). In the present report, applicants found that IL-10 antisense not only inhibits the growth but and also lead to cell death of malignant B-1 cells in some patients with chronic lymphocytic leukemia.

The effect of IL-10 antisense was manifested in a time and dose dependent manner. As reported by others (82, 83), the effect of antisense increased with time. In our experiment, the inhibitory effects were maximal at a concentration of 20μm, at 72 hours after addition of the IL-10 antisense oligos to culture. Sufficient time may be required for the cells to take up the antisense oligos and for the translation of IL-10 protein to be reduced. In addition, enough time must be allowed for the cell to deplete residual IL-10 protein formed prior to the addition of antisense oligos.

In summary our results showed that IL-10 may be an autocrine growth factor for malignant B-1 cells and that antisense therapy directed at IL-10 may be a potential tool in modulation of some B cell leukemias.

Throughout this application, various publications have been referenced. The disclosures in these publications are incorporated herein by reference in order to more fully describe the state of the art.

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While the invention has been particularly described in terms of specific embodiments, those skilled in the art will understand in view of the present disclosure that numerous variations and modifications upon the invention are now enabled, which variations and modifications are not to be regarded as a departure from the spirit and scope of the invention. Accordingly, the invention is to be broadly construed and limited only by the scope and spirit of the following claims.

We claim:

1. A method for treating a patient with a disease in which the levels of interleukin-10 need to be down-regulated which comprises adminstering to the patient a therapeutically effective amount of an antisense oligodeoxynucleotide specific for interleukin-10 mRNA.

2. The method according to claim 1, wherein the disease is a disease in which interleukin-10 is an autocrine growth factor for malignant cells or a disease in which the inflammatory response is suppressed.

3. The method according to claim 2, wherein the disease is a disease in which interleukin-10 is an autocrine growth factor for malignant cells.

4. The method according to claim 2, wherein the disease is aquired inmmunodeficiency syndrome.

5. The method according to claim 2, wherein the malignant cells are B-1 cells.

6. The method according to claim 5, wherein the disease is chronic lymphocytic leukemia.

7. The method according to claim 1, wherein the antisense oligodeoxynucleotide specific for interleukin-10 mRNA spans the region adjacent to the initiation site of interleukin-10 translation.

8. The method according to claim 7, wherein the antisense oligodeoxynucleotide specific for interleukin-10 mRNA spans the region adjacent to the initiation site of interleukin-10 translation, region 1-500.

9. The method according to claim 8, wherein the antisense oligodeoxynucleotide specific for interleukin-10 mRNA spans the region adjacent to the initiation site of interleukin-10 translation, region 310-347.

10. The method according to claim 9, wherein the antisense oligodeoxynucleotide specific for interleukin-10 mRNA spans the region adjacent to the initiation site of interleukin-10 translation, region 315-342.

11. The method according to claim 10, wherein the antisense oligodeoxynucleotide is 5'-TGGGTCTTGGTTCTCAGCTTGGGGCAT.

12. A method for treating a patient with chronic lymphocytic leukemia which comprises adminstering to the patient a therapeutically effective amount of an antisense oligodeoxynucleotide specific for interleukin-10 mRNA.

13. The method according to claim 12, wherein the antisense oligodeoxynucleotide specific for interleukin-10 mRNA spans the region adjacent to the initiation site of interleukin-10 translation.

14. The method according to claim 13, wherein the antisense oligodeoxynucleotide specific for interleukin-10 mRNA spans the region adjacent to the initiation site of interleukin-10 translation, region 1-500.

15. The method according to claim 14, wherein the antisense oligodeoxynucleotide specific for interleukin-10 mRNA spans the region adjacent to the initiation site of interleukin-10 translation, region 310-347.

16. The method according to claim 15, wherein the antisense oligodeoxynucleotide specific for interleukin-10 mRNA spans the region adjacent to the initiation site of interleukin-10 translation, region 315-342.

17. The method according to claim 16, wherein the antisense oligodeoxynucleotide is 5'-TGGGTCTTGGTTCTCAGCTTGGGGCAT.

18. An antisense oligodeoxynucleotide specific for interleukin-10 mRNA having the formula 5'-TGGGTCTTGGTTCTCAGCTTGGGGCAT.

FIGURE 1

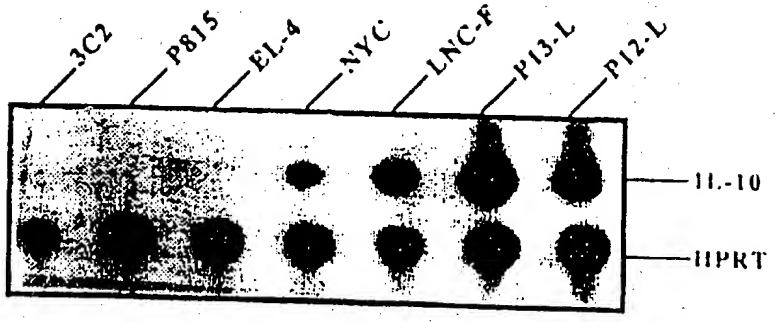

FIGURE 2

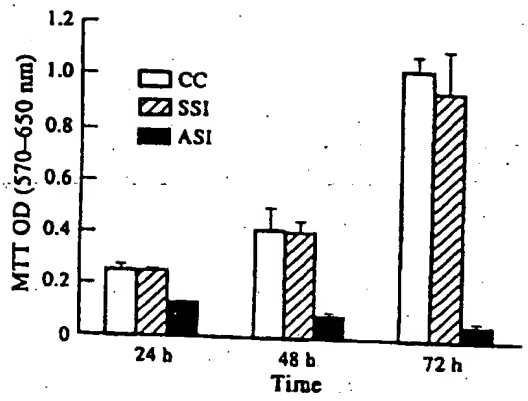

FIGURE 3

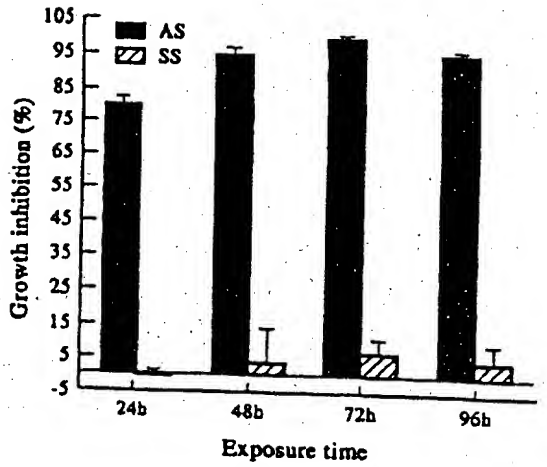
AS
SS
Growth inhibition (%)
105
95
85
75
65
55
45
35
25
15
5
-5
24h
48h
72h
96h
Exposure time

FIGURE 4

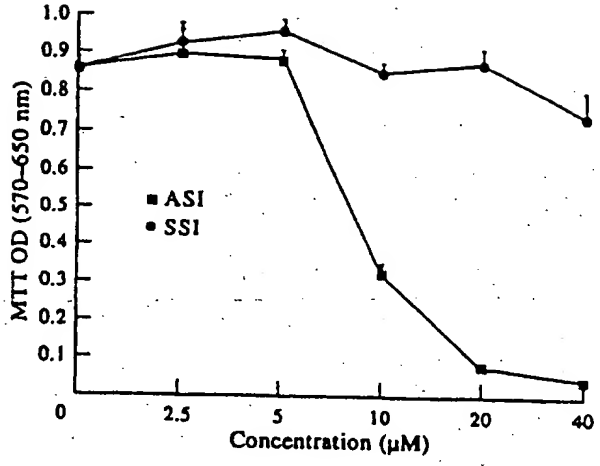
MTT OD (570–650 nm)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ASI
SSI
2.5
5
10
20
40
Concentration (μM)

FIGURE 5

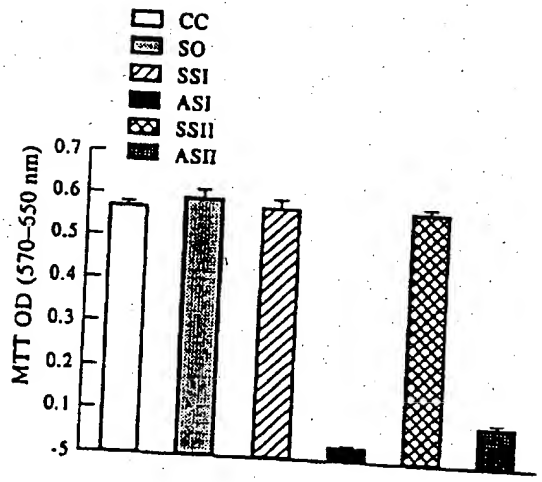

FIGURE 6

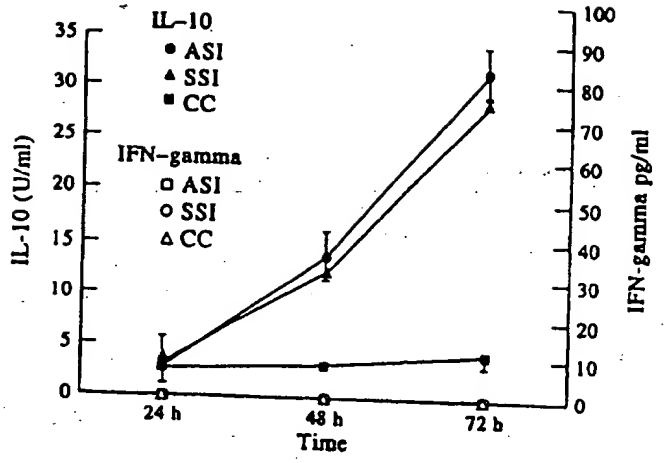
IL-10
ASI
SSI
CC
IFN-gamma
ASI
SSI
CC
IL-10 (U/ml)
IFN-gamma pg/ml
0
5
10
15
20
25
30
35
0
10
20
30
40
50
60
70
80
90
100
24 h
48 h
72 h
Time

FIGURE 7

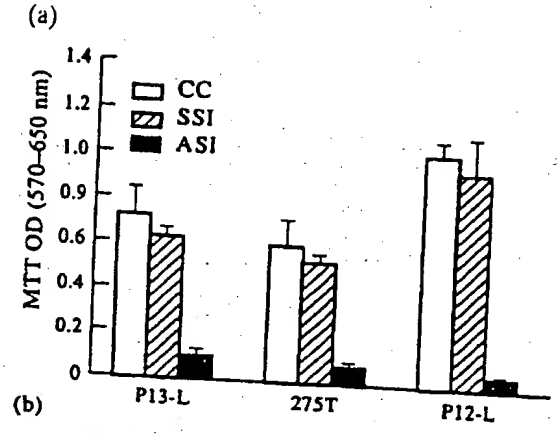

Fig. 7A

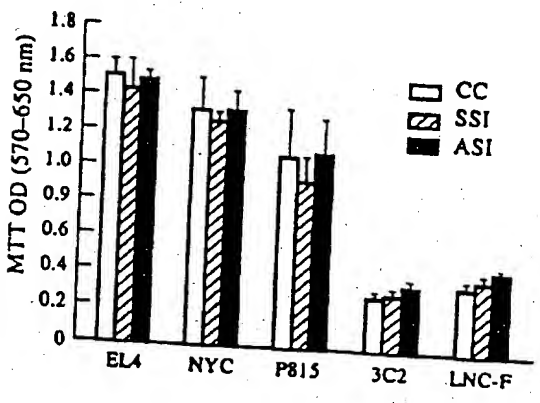

Fig. 7B

FIGURE 8

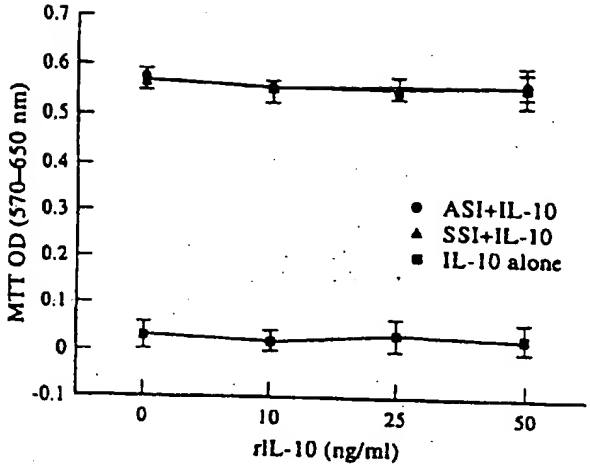
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
MTT OD (570–650 nm)
ASI+IL-10
SSI+IL-10
IL-10 alone
0
10
25
50
rIL-10 (ng/ml)

FIGURE 9

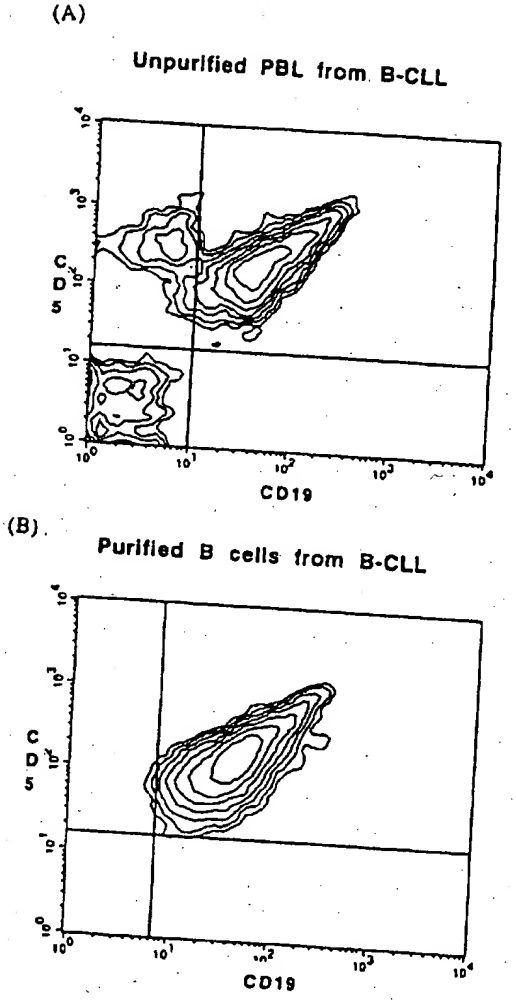

Fig. 9A

Fig. 9B

FIGURE 10

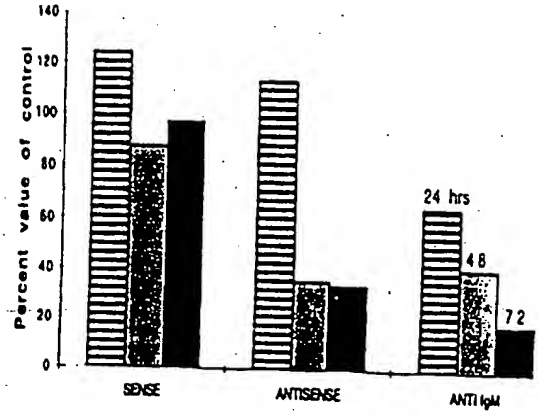
Percent value of control
140
120
100
80
60
40
20
0
24 hrs
48
72
SENSE
ANTISENSE
ANTI IgM

FIGURE 11

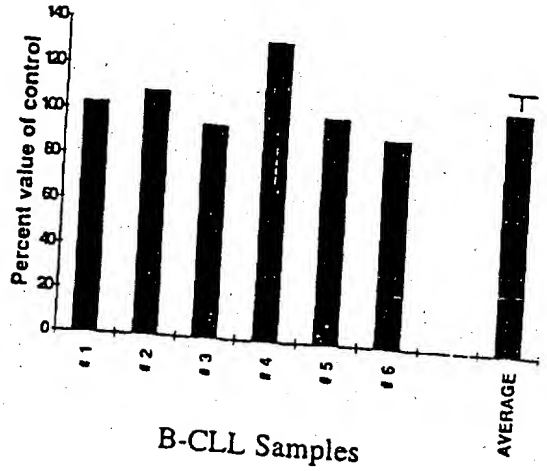

FIGURE 12

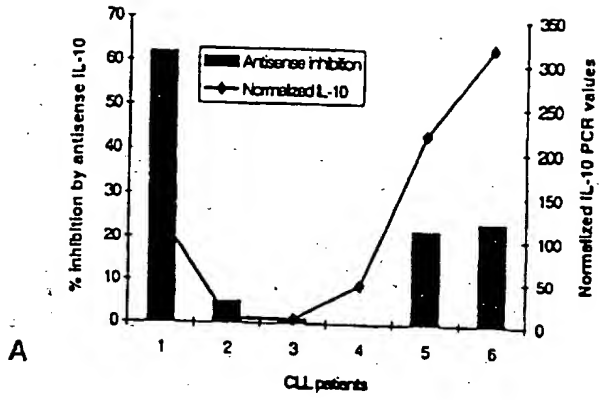

Fig. 12A

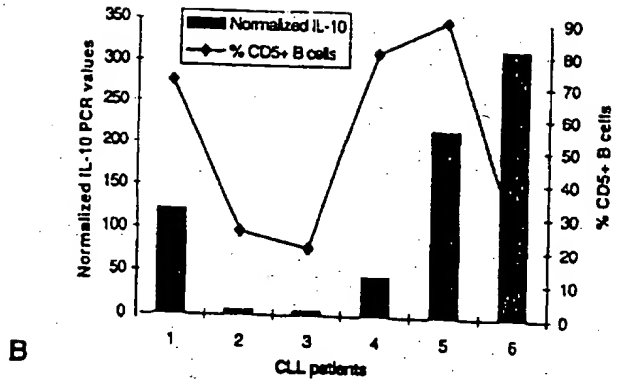

Fig. 12B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/03244

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01N 43/04, 63/00, 65/00; A61K 31/70, 48/00; C07H 21/04
US CL :514/44; 536/24.5; 424/93.1, 93.21
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 536/24.5; 424/93.1, 93.21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG: MEDLINE, EMBASE, BIOSIS, DERWENT; APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MASOOD et al. Interleukin-10 is an Autocrine Growth Factor for Acquired immunodeficiency Syndrome-Related B-Cell Lymphoma. Blood. 15 June 1995, Vol. 85, pages 3423-3430, see entire document.	1-18
A	JAMES, W. Towards gene-inhibition therapy: a review of progress and prospects in the field of antiviral antisense nucleic acids and ribozymes. Antiviral Chemistry & Chemotherapy. 1991, Vol. 2, No.4, pages 191-214, see entire document.	1-18
A	STULL et al. Antigene, Ribozyme and Aptamer Nucleic Acid Drugs: Progress and Prospects.Pharmaceutical Research. 1995, Vol. 12, Number 4, pages 465-481.	1-18

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
14 APRIL 1997

Date of mailing of the international search report
30 MAY 1997

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
ABDUR RAZZAQUE

Telephone No. 703-308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/03244

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GURA, T. Antisense Has Growing Pains. Science. 27 October 1995, Vol. 270, pages 575-577, see entire document.	1-18